Field of invention

The present invention is a method of designing and preparing a nanoparticle system for combination chemotherapy, to be used for cancer treatment. In particular, two anti-cancer agents are loaded simultaneously in different compartments nanoparticulate system to be delivered to the target site in a non-invasive manner. The two drugs will target different metabolic pathways and hence provide better treatment efficacy.

Background of invention

Cancer or related disorders are generally characterized by rapidly proliferating cells such that the growth of these cells is uncontrolled. Conventional cancer treatments include surgery, radiation therapy or chemotherapy. Surgery is helpful only in cases where tumor is localized or early stages of cancer, but in most of the patients, combination of these therapies have to be used to control the tumor growth and still the survival rates are low. Chemotherapy is chosen in cases of metastasized tumors or later stages of tumors, but it has many side effects associated with it. Combination of two or more chemotherapeutic agents may be useful in reducing the tumor as multiple molecular mechanisms can be targeted which reduces the chances of drug resistance, but the side effects become more severe. So, there is a need to increase the effectiveness and eliminate or reduce the side effects associated with conventional chemotherapy.

Two main reasons for the decreased efficacy of chemotherapeutic agents given conventionally are lack of target specificity due to systemic circulation of the drug and lack of retention of the drugs in the target tumor site. Nanoparticles can address these issues by increasing the circulation time and better uptake by tumor tissues by EPR effect.

As tumor growth and metastasis is dependent on formation of new blood vessels i.e. angiogenensis, the anti-angiogenic agents are also being used for cancer therapy. Studies have

shown that combination of chemotherapeutic agent with anti-angiogenic agent increases the efficacy of treatment. Such combination therapy has been disclosed in multiple patents like U.S. Patents 6,147,060; 5,856,315; 5,731,325; and 5,574,026. None of these deal with a nanoparticle for co-delivery of two drugs. The patents deal with direct sequential injection of two agents as free drugs.

US patent 20100303912 A1, describes the nanoparticles with PLGA core linked to doxorubicin surrounded by lipid shell consisting of PC, cholesterol and DSPE-PEG entrapping combretastatin A4 (anti-angiogenic agent). But in the proposed formulation doxorubicin is covalently linked to the polymer, the amide bond conjugation might affect the therapeutic efficacy. Also, the method of PLGA activation and its conjugation to doxorubicin is a very complex process wherein the present invention involves simple preparation method at room temperature.

US patent 20100112077 A1, nanoparticles of paclitaxel and albumin (abraxane) is given in combination with bevacizumab (anti-VEGF antibody). In this invention the Paclitaxel is given in the nanoparticles formulation intravenously, but the anti-angiogenic agent bevacizumab is given separately as i.p injection. Hence the patent does not teach a nanoparticle complex for codelivery of two drugs, rather combines two single drugs as individual modalities given one after the other. In the present invention, both the drugs are loaded in the same delivery vehicle, which gives the advantage of having control over drug loading and release kinetics.

US patent 7,850,990 issued to Tardi et al., describes combination of cisplatin with different anticancer agents like daunorubicin, topotecan, irinotecan, 5-fluorouracil and vinorelbine encapsulated in liposomes with different compositions of phospjholipids combined with cholesterol to achieve synergism of the two drugs in vivo for a range of concentrations. But, encapsulation efficiency of these drugs in the liposomes is not considered, as loading of hydrophilic drugs in liposomes is quite low. Also the loss of drug during extrusion is not accounted. The patent deals with both drugs being entrapped in the same lipid vesicle, resulting

in low encapsulations, does not deal with combined lipopolymeric nanostructures. Further, the patent includes cholesterol in the liposomes which may have undesirable effects in the body.

Summary of invention

A major objective of the present invention is to prepare a nanoscale delivery system which can co-encapsulate a hydrophobic and a hydrophilic agent and can be given as aerosols via inhalation route for direct delivery to lungs. Another objective is to provide sustained release of the encapsulated drugs through the invented formulation. Yet another object is to provide a delivery vehicle which is biocompatible, biodegradable and can withstand the shear forces during nebulization so that it becomes suitable for aerosol delivery. Another object was to exploit the combination of anti-angiogenic agent dopamine with a chemotherapeutic agent. Advantages of the invention include simple preparation at room temperature, co-encapsulation of hydrophobic and hydrophilic drug in different compartment of the same delivery system so that they can be delivered simultaneously. The invented formulation maintains airway patency and shows a higher alveolar deposition. Higher cellular internalization by endocytic pathway and improved cytotoxicity in lung cancer cell lines is shown by the novel formulation.

Brief Description of the invention

This invention relates to a nanohybrid system involving a lipid nanovesicle conjugated to a polymeric nanoparticle through a covalent bond. Lipids used in this case may involve phospholipids like phosphatidylcholine, phosphatidylethanolamine, phosphatidylseine, in the absence of cholesterol, and are linked by peptide bonds to a nanoparticle of biopolymers like alginate or chitosan.

The invention is a system for delivery of a hydrophobic and a hydrophilic drug in two compartments of the nanoscale delivery vehicle. Nanovesicles show a high encapsulation of

lipophilic drugs and hence can be the carrier for the same, while the encapsulation of hydrophilic drugs in liposomes is very low, so such drugs can be loaded in the polymeric nanoparticles.

The nanohybrids encapsulate two drugs, one which is an anticancer drug like paclitaxel, docetaxel, camptothecin etc and the other is an antiangiogenic agent like dopamine, anti-VEGF antibody.

The preparation of the nanohybrid system is a two step process. The nanovesicle and the polymeric nanoparticles have to be synthesized separately and then coupled together by EDC linkage. As the amide bond is formed between free carboxyl group on the surface of alginate nanoparticles and free amino group on the surface of the DOPE present in lipid nanovesicles. Liposomes are prepared by thin film hydration or ethanol injection method and hydrophobic drug like Paclitaxel is added to organic solvent in which lipids are dissolved, in the ratio of 1:2 molar ratio while the preparation of thin film, which is then hydrated to get 2 mg/ml of final lipid concentration. This results in the formation of multilamellar vesicles which are sonicated to get desired unilamellar vesicles of DPPC:DOPE with the size ranging from 200 – 250 nm. Alginate nanoparticles are prepared by controlled ionic gelation method and the hydrophilic drug dopamine is added in ratio of 1:2 (w/w) in the alginate solution before addition of calcium chloride solution. The mixture is kept overnight and then centrifuged to separate the nanoparticles at 19,000 rpm for 45 minutes. For preparation of the invented nanohybrid system, the alginate nanoparticles are mixed with the DPPC:DOPE nanovesicles and to this mixture EDC and NHS are added and reacted for 2 hours at room temperature. Then mixture is centrifuged and pellet is resusupended in the buffer to form the nanovesicle-biopolymeric nanoparticle hybrids.

The present invention had shown high encapsulation efficiency of the loaded drugs. For example, encapsulation of Paclitaxel was 85.4 ± 4.2 % (n=3) and of dopamine was 41.6 ± 2 % (n=3). The drugs have shown sustained release of the drugs from the system with around 8% release of Paclitaxel in 10 hours and subsequent sustained release over period of 72 hours.

Similarly sustained release of dopamine was observed followed by an initial burst release.

Since the formulation is prepared to be given as aerosol, thus the airway patency and *in vitro* lung deposition is also studied. Drug loaded formulation had shown high airway patency of around 97% as compared to free dugs which remains less than 5% as in the case of taxol. It implies that the surfactant properties of the formulation are not affected by loading of drugs. *In vitro* deposition study carried out using twin impinger had shown maximum deposition in stage II, indicating the potential of the formulation to reach the terminal airways.

In vitro anti-tumor efficacy of the formulation is evaluated in A549 (non small cell lung carcinoma) and NCI-H460 (human lung cancer cell line). IC50 of the formulation was calculated after 48 hours of exposure as shown in figure 7. As can be seen, the formulation containing Paclitaxel shows significantly less IC50 as compared to Paclitaxel alone indicating advantage of the invented formulation.

Cellular uptake study for the present invention is carried out using formulation which is prepared by loading nile red in the liposomes and calcein in the alginate nanoparticles. The A549 cells have shown bright fluorescence in the presence of this formulation due to uniform colocalization of the dye loaded nanoparticles inside the system. In presence of free dye, less/no fluorescence is observed as seen in figure 8. Also it was seen that cellular uptake of the formulation is inhibited in the presence of 0.1% azide, implying that uptake of formulation is ATP dependent as azide being a metabolic inhibitor depletes the cell of ATP. By studying the uptake of formulation in presence of different endocytic inhibitors, it was obtained that phenothiazine inhibited cellular internalization indicating that the clathrin mediated pathway is involved in the endocytosis of the invented formulation.

As the invented formulation simultaneously target two pathways involved in cancer development, thus it has potential to show improved anti cancer efficacy *in vivo*. Also, different

routes of administration other than aerosol, like intravenous and intraperitoneal can be exploited for this formulation. Further, a targeting moiety like a ligand, or antibody can be attached to the invented formulation for better reach at the target site.

The invented formulation can be used for delivery of different combinations of hydrophilic and hydrophobic drugs. So, different anti-angiogenic agents like sorafenib, sunitinib, everolimus and similar drugs. Different chemotherapeutic agents can also be used like doxorubicin, docetaxel.

Examples

Example 1

Preparation of paclitaxel loaded liposomes

DPPC:DOPE (6:4 molar ratio) liposomes were prepared by thin film hydration method. The calculated quantity of lipids were weighed and dissolved in chloroform and methanol mixture (2:1) in a round bottom flask. Paclitaxel was added to this lipid mixture in the molar ratio of 1:2. Thin film was formed by evaporating the solvent under vacuum, for 15 minutes at a temperature of 40°C, using rotator evaporator. The film was hydrated for 1 hour with TES buffer at 160 rpm and temperature of 50°C. Volume of TES buffer is kept such that final concentration of lipid is 2mg/ml. Then, the prepared suspension is then sonicated using probe sonicator at 50% amplitude, for 2 minutes to get small unilamellar vesicles.

Example 2

Preparation of alginate nanoparticles Alginate nanoparticles were prepared by cation induced controlled gelification of sodium alginate. 10ml of 0.06% (w/v) solution of sodium alginate was prepared in Milli-Q water. 0.5ml CaCl2 (18mM) was added to 9.5 ml of sodium alginate solution containing 6mg of dopamine. Then 2 ml of chitosan solution (0.05%) was added to the mixture

and it was stirred at room temperature for 30 minutes at 1000 rpm. The mixture is then kept overnight at room temperature. Nanoparticles are obtained by centrifugation at 19,000 rpm for 45 minutes and the pellet is washed once and then resuspended in Milli-Q water.

Example 3

Preparation of nanohybrid system for dual delivery For the preparation of EDC coupled nanohybrid systems, a liposomal suspension of DPPC:DOPE (6:4 ratio) is prepared in TES buffer. An aliquot of the alginate nanoparticles solution is then added to the liposomal suspension. 100mg EDC and 50mg N-hydroxy succinimide (NHS) were then added to this nanoparticles mixture. The carbodiimide was solublized using a vortex mixer. The mixture was reacted for 2 hours at room temperature for the coupling of free –NH2 group of the liposomes to form amide linkage with the free –COOH groups of the alginate nanoparticles. Then the mixture was centrifuged at 25000g for 25 minutes and the supernatant of the centrifuged solution was discarded and pellet was washed once before resuspending it in TES buffer. The resulting solution was then sonicated and kept at 4°C till further use.

Example 4

Particle size and zeta potential determination

The average size of the individual nanoparticles was found to be in the range of 150-250 nm, while the size of the nanohybrid ranges from 400-450 nm as determined by dynamic light scattering (DLS) using laser particle analyzer (BI 200SM, Brookhaven Instruments Corporation). Also the polydispersity of the formulation is low indicating the uniform size distribution (Table 1). Zeta potential of the final formulation was around -20mV as measured by ZetaPALS (Brookhaven Instrument Corporation, USA) (Figure 1).

Example 5

Transmission electron microscopy (TEM) of nanoparticles

The size of the formulation is further confirmed by TEM, which also helped in understanding the

morphology of the novel nanoparticles. TEM analysis of the nanoparticles formulations was done according to the negative staining method. 0.5L of each of the nanoparticle formulation was mixed with equal volume of 1% solution of PTA (phosphotungstic acid, H3PW12O). PTA was used because it provides contrast to the image. The resultant solution was then vortexed for about 10-15 seconds for its proper mixing and then kept still for fixation for about 2 minutes. A drop from the mixture was then placed on the carbon coated copper grids and then blotted dry. The grids were subsequently subjected to negative staining by 2% aqueous solution of uranyl acetate (UO2(CH3COO)2.2H240O) and air dried. Dried samples were then analyzed by transmission electron microscope (model: CM200, Philips) operating at 120kV (Figure 2).

Example 6

Contact angle measurement

The contact angle measurement studies were done with goniometer (KSV Cam 100 Optical Contact Angle Meter, version 1.0, KSV Instruments Ltd.). The contact angle measurement studies were done with water dropped over the glass slides on which films of various formulations were made. It can be inferred from the results that both the nanoparticles and the novel conjugate have hydrophilic surface as all of them have shown contact angle values less than 40°. Alginate nanoparticles are highly hydrophilic with contact angle of 16.89°. The invented formulation has contact angle less than corresponding liposomes, it indicates the change in surface properties after EDC linkage with alginate nanoparticles with relatively lower contact angles (Figure 3).

Example 7

FTIR analysis

FTIR analysis of the lyophilized formulations without drug is carried out to confirm the amide bond formation between carboxyl group on the surface of alginate nanoparticles and primary amine groups of liposomes. The high intensity of absorbance at *1650.78 cm* in the nanohybrid system attributes to the stretching of secondary amide may attribute to the formation of amide

bonds between the functional groups on surface of liposomes and alginate nanoparticles (Figure 4).

Example 8

Drug encapsulation efficiency determination

The invented formulation has shown encapsulation of both hydrophobic and hydrophilic drug. Present invention was found to have a high encapsulation efficiency of 85.4 ± 4.2 % (n=3) for Paclitaxel and 41.6 ± 2 % (n=3) for dopamine as determined by UV-spectrophotometry (Perkin Elmer Lambda 25) at 228 nm and 280 nm respectively.

Example 9

In vitro drug release study Release studies are performed by dialysis bag method in USP dissolution apparatus type-II (Electrolab, TDT-08L). Dialysis was done by using dialysis membrane-50 (molecular weight cutoff 5000-10000) from himedia, which were kept soaked in double distilled water for 12 hours prior to use. 3ml of each formulation was poured in different bags and both the ends are sealed by thread. The sealed bags were placed in 150ml of release medium in dissolution vessels at 37± 2°C. The release medium was stirred at 150 rpm to enhance the solubility of the drugs and prevent the formation of a stagnant layer at the membrane and outer solution surface. 2 mL aliquots were taken from the medium at different predefined intervals and replaced with an equal volume of fresh medium. This replenishing assured the maintainance of good sink conditions. Amount of drug in the withdrawn aliquots was quantified by UV-spectrophotometry at appropriate dilutions. The main constraint in studying the release behavior of the two drugs simultaneously from the dual drug loaded nanohybrid system is the overlap of the UV-Vis maxima for the two drugs is in the range of 220-280nm. To work around this problem, two formulations of the nanohybrid systems are prepared. The first one is normal system containing both DA and PTX. In this formulation, release of DA was studied. The second formulation comprised empty alginate nanoparticles and liposomes containing PTX to facilitate the study of release of PTX. Paclitaxel have shown a sustained release over a period of 72 hours,

while there is burst release of dopamine with around 40% release in 10 hours (Figure 5).

Example 10

Airway patency study

As the present formulation is intended for aerosol administration, it was crucial to evaluate its surface activity and hence the airway patency. Capillary surfactometer (CS) from Calmia Biomedicals (Toronto, Ontario) was used to study the airway patency and surface ability of formulations. Briefly, 0.5 µl of the sample to be evaluated is introduced into the narrow section of the glass capillary, where the internal diameter is 2.5 mm, similar to the terminal airway. Capillary, at its one end is connected to bellows and pressure transducer. Pressure is raised and this increasing pressure results in the passage of the sample from the narrow section of the capillary, allowing air to get through it which results in the abrupt lowering of the pressure. If the sample contains well functioning pulmonary surfactant the sample liquid will not return to the narrow section and the capillary remains unoccluded. If on the other hand, the sample does not contain well functioning pulmonary surfactant the sample liquid will return repeatedly resulting in significant resistance to the airflow. The airway patency of the formulation was studied as % opening time of capillary over an observation period of 120 seconds. It was found that the % opening time of capillary remains greater than 97% even after drug loading, which indicates that surfactant properties of the formulation is not affected by drug loading and is better as compared to taxol (Figure 6).

Example 11

In vitro lung deposition study

In vitro lung deposition studies of formulation was performed using glass twin impinger apparatus (Copley Scientific, Nottingham, UK), adapted from apparatus A of European and British Pharmacopeia. The twin impinger comprises of two stages and is attached to a vacuum pump with as adjustable air flow rate which can disperse the aerosol inside the impinger. The upper stage (stage I) represents the upper airways or the tracheobronchoial region and the lower

stage (stage II) represents the lower airways or the alveolar region. There is a region at the top of the impinger which simulates the throat region. The deposition of the present formulations was studied in stage I and II. The nanoparticles suspension was placed in the sample holder and nebulized using Omron Micron AIR U22 ultrasonic nebulizer. 60 L/min air flow rate was maintained inside the impinger during nebulisation by vacuum pump. In the impinger, 7 and 30 ml of acidified methanol (200µL of glacial acetic acid added to 1 L methanol) was taken in upper and lower chambers respectively. Nebulization was done for 5 minutes and at the end samples were collected from stage I and II and then quantified for paclitaxel using UV spectrophotometer. Deposition in stage II is much higher as compared to stage I for all the formulations, indicating the potential of the formulation to reach the terminal airways (Figure 7).

Example 12

In vitro cytotoxicity assay

In vitro cytotoxicity analysis of the formulation was done using A549 (non small cell lung carcinoma) and NCI-H460 (human lung cancer cell line) purchased from National Centre for Cell Sciences, Pune, India. Cells were routinely maintained in 75 cm4 cell culture flasks with DMEM containing 10% foetal bovine serum and 1% antibiotic penicillin/streptomycin mix. Cells were trypsinized by trypsin-EDTA solution and cells were counted. 10 cells are added per well in 96-well microtiter tissue culture plate (NUNC, USA) and incubated in CO2 incubator to allow adherence. After 24 hours, the samples were added in 7 different concentrations of each different formulations ranging from 10 nM to 10 µM of culture medium. After 48 hours, SRB assay was conducted to determine the cell viability. IC50 value of the formulation was much less than Paclitaxel alone dissolved in DMSO, which indicates advantage of the invented formulation (Figure 8). The formulation without drug did not show cytotoxicity even at a high concentration of 10 µM.

Example 13

Cellular uptake and internalization

Cellular uptake and internalization studies were done by loading nile red dye in the liposomes and calcein in alginate nanoparticles at concentration of 0.5 mg/ml and then studying the uptake and internalization of these nanohybrid systems by A549 cells. 70 – 80% confluent cells were seeded on glass coverslips placed in 24-well tissue culture plate at a density of 10 cells per well as 1 ml volume of the suspension. Plate was incubated at 37°C at 5% CO2 incubator for 24 hours. Spent medium from the wells was then replaced by fresh medium containing dye loaded formulations. Following incubation of 3 hours, medium was removed from the wells and washed with phosphate buffered saline (PBS) to remove untrapped dye. 1 ml of 10% formaldehyde solution in PBS was then added to each well and was retained there for 10 minutes in order to fix the cells that were adhered on the surface of the coverslips. Coverslips were then placed on the glass slide over glycerol mount in order to prevent the cells from drying. Slides were observed by a confocal laser scanning microscope (CLSM) (Olympus Fluoview, FV500, Tokyo, Japan) using an excitation wavelength of 570 nm and emission wavelength of 590 nm for nile red and using an excitation wavelength of 503nm and emission wavelength of 525 nm for calcein. Images were acquired and analyzed with 60X oil immersion objective using the Fluoview software (Olympus, Tokyo, Japan).

For determination of endocytic pathway, the protocol mentioned above is used. The endocytic inhibitors Nystatin (2µg/ml), Colchicine (4µg/ml), Phenothiazine (10µg/ml) and Azide (10µg/ml) were added to the cells and incubated for 1 hour prior to sample addition. And then the subsequent steps are followed for CLSM analysis. It is clearly indicated from the CLSM images that the least fluorescence is exhibited with azide and phenothiazine (Figure 9). It implies that uptake of the nanoparticles is ATP dependent and also that the formulation is internalized by clathrin mediated endocytosis.

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Figures

Nanoparticle	Mean diameter (nm)	Polydispersity Index
DPPC: DOPE nanovesicle	140.4±5.5	0.187±0.02
DPPC:DOPE nanovesicle + paclitaxel (PTX)	174.4±9.8	0.21±0.01
Alginate nanoparticles	238.5±33.4	0.2±0.027
Alginate nanoparticles + dopamine (DA)	284.02±34.25	0.221±0.03
Invented formulation: PE-ALg nanohybrid	435.2±32.6	0.19±0.05
Drug loaded invented formulation: PE-Alg nanohybrid + dopamine + PTX	480.2±37	0.22±0.15

Table 1. DLS results of the nanovesicle-polymeric naohybrids (n=3)